LESSON 26:
GERMPLASM PRESERVATION : CRYOBIOLOGY

Introduction

What are Threatened Plants?

In the recent years, with the tremendous increase in the population, pressure on the forest and land resources have increased which has resulted in the decline in the population of medicinal and economically important plant species. Even some of the plant species are at the verge of vanishing because of severe threat to their natural habitat due to human interference. Such species are termed as 'threatened' species.

Thus, attempt has been made in the recent years at the national and international levels to protect and preserve plant species, threatened plants as well to those plant species which are not in use today but may serve as important resource for future breeding programme.

In India, Botanical Survey of India has released three volumes (Red Data Book) enlisting the threatened plants of Indian subcontinent. There are several other organisations, which are engaged in the protection and preservation of su.ch plants. Some of these organisations are Indian Council of Agricultural Research, New Delhi; International Bureau of Plant Genetic Resources, Nottingham, U.K.; National Bureau of Plant Genetic Resources, Pusa Campus, New Delhi; Council of Scientific and Industrial Research, New Delhi and Botany Departments of various Universities. Attempts have been made to conserve the germplasm by preserving the genetic material, which can be done by two ways:

A. In-situ preservation

B. Ex-situ preservation.

A. In Situ Preservation

It aims at the preservation of the germplasm in their natural environment by establishing

biosphere reserves, national parks, gene sanctuaries etc.

Limitations: The limitation of this type of preservation is the risk of declination of the preserved species due to environmental hazards.

B. Ex Situ Preservation

This is the chief mode of preservation of germplasm. Providing the suitable condition in the gene bank preserves the genetic materials in the form of seed or in vitro cultures. But for being successful in establishment of gene bank considerable knowledge of genetic structure as well as elements influencing them are necessary.

Usually seeds form the most common material to conserve plant germplasm in the seed propagated plants. But this method has certain limitations such as:

- Loss of seed viability with passage of time.
- Seed destruction due to seed born pathogens, pest etc.
- This method is confined only to seed propagating plants.

In contrast to seed propagating plants, the vegetatively propagated plants are preserved in vitro as shoots, meristems, embryos etc.

The advantages of in vitro preservation over in situ preservation are

- Large amount of material can be preserved in small area.
- It overcomes the destruction due to environmental hazards.
- It provides large amounts of plant material for culturing.

Using either of the following techniques, (Fig.1.), preservation of germplasm can be achieved.

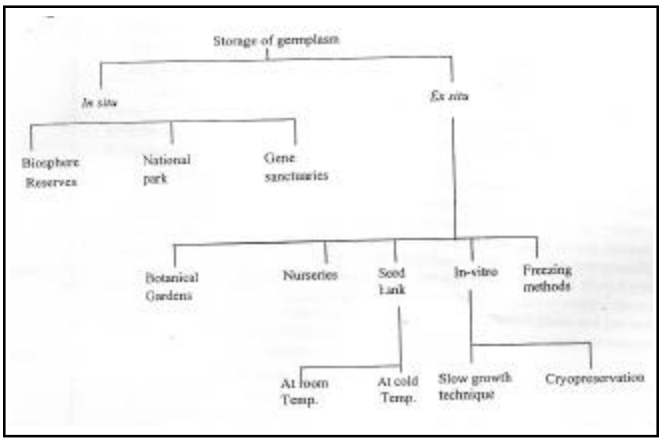

Fig.1. Various approaches for Germplasm preservation

In this chapter, details of germplasm preservation by cryopreservation (cryo means extreme cold, derived from Latin word kruos = 'frost') using in vitro grown materials are presented.

Cryopreservation

What is Cryopreservation?

Cryopreservation (preservation in the frozen state) is based on the reduction and subsequent arrest of metabolic functions of biological material by imposition of ultra-low temperature. At the temperature of liquid nitrogen (-196°C) almost all the metabolic activities of cells are ceased and the sample can then be preserved in such state for extended periods. However, only few biological materials, in their natural state, can be frozen to sub-freezing temperatures without adversely affecting the cell viability. Knowledge about the chemicals having cryopreservative properties such as glycerol and dimethyl sulfoxide facilitated the development of effective cryopreservation technique.

One of the earliest records on use or cryopreservation technique is of Sun (1958), who attempted to preserve desiccated seedlings of Pisum sativum. With the rapid development in plant cell, tissue, and organ culture and regeneration through

various explants, emphasis has laid to cryopreserve the genetic resources for their use in the future.

A. Need for Cryopreservation

The main application and objective of developing cryopreservation technology is preservation of valuable genetic resources, especially of vegetatively propagated and also of those species which have short lived seeds. Therefore, it is imperative that use of cryopreservation technology requires efficient regeneration protocols through tissue culture of the species. Cryopreservation of endangered species is one of the most important objectives of NBPGR, New Delhi. Endangered species for which micropropagation techniques have been developed in India are listed in Table1. It has been recommended that the regeneration methods using apical meristem is advantageous as these are simple and there are no chances of genetic variation. It is essential that tissue culture methods do not create genetic variability.

Table1. Threatened species for which micropropatation methods have been developed.

Boswellia serrata	Commiphora wightii
Calligonum polygonoides	Coptis teeta
Caralluma spp	*Nepenthes khasiana*
Ceropegia bulbosa	Podophyllum hexandrum
Chlorophytum borivilianum	Tecomella undulat

In contrast to above, the cases where genetic variability has been induced in the cultures, this variability need preservation for use in the future. Therefore, cryopreservation technique is equally good for preserving the genetic resources of existing genotypes and also of new variants.

B. Procedure of Cryoprotection and Pretreatment

Some form of cryoprotection is necessary for cryopreservation of plant material unless they are naturally dehydrated, as in the case of dormant vegetative buds in the winter, or artificially cold acclimated. Several chemicals such as dimethyl sulphoxide (DMSO), glycerol, various sugars and sugar alcohols protect living cells against damage during freezing and thawing (means to become unfrozen or warm after preservation at ultra low temperatures). These compounds lower the temperature at which freezing first occurs and can alter the crystal habit of ice when it separates. The colligative properties of the cryoprotectants minimize the harmful action of electrolyte concentration resulting from conversion of water into ice. High solubility in aqueous phase and low toxicity to the cells are the two essential characteristics for cryoprotectants.

Cryoprotectants are categorised as

1. Permeating, e.g., DMSO, methanol, glycerol and
2. Non-permeating, e.g., sugars, sugar alcohols, high molecular weight polymers as dextran, polyvinyl pyrrolidone, hydroxy ethyl starch.

The cells require different pretreatment periods with different compounds for proper cryoprotection. DMSO enters more rapidly than glycerol, in permeating types, and therefore requires shorter period for treatment. Most of the cryoprotectants exhibit varying degree of cytotoxicity at higher concentrations. Generally, DMSO at 5 to 10% and glycerol at 10 to 20% are used as cryoprotectants. Sometimes, a mixture of cryoprotectants can also be used to improve the efficacy. Addition of osmotically active compounds in the culture medium such as mannitol, sorbitol, sucrose and proline increase the freezing resistance of the cells. These compounds mainly act by their dehydration effect. Mannitol has been found to be beneficial in reducing the mean cell volume of cells of Acer pseudoplatanus and Capsicum annuum and increase their post-freezing survival. Therefore, all such compounds also possess cryoprotective properties besides that they are osmotically active. Sorbitol has been successfully used as an osmotic agent as well as cryoprotectant for Glycine max, Datura innoxia, Brassica napus, and Daucus carota.

In a detailed study using nuclear magnetic resonance (NMR) and differential thermal analysis (DTA), it was recorded that incorporation of DMSO or sorbitol into the liquid medium had a significant effect in the temperature range for initiation to completion of ice crystallization and the amount of liquid water present in the cells at various sub zero temperatures. A close association between the percent-unfrozen water at -40oC and the percent cell survival after freezing in liquid nitrogen was observed. These results explain the cryoprotective properties of DMSO and sorbitol and showed that a combination of DMSO and sorbitol was more effective than use of single compound as cryoprotectant. The beneficial effect of using a mixture of cryoprotectants involving PEG, glucose and DMSO (10:8:10%) has been demonstrated in sugarcane callus.

Cold hardening or abscisic acid (ABA) treatment of wheat suspension culture increased the freezing resistance and survival following cryopreservation. Similarly, cold hardening of Panax cell suspension cultures at 2°C in a medium supplemented with 3 to 25% sucrose increased the survival of cells after storage in liquid nitrogen. Incorporation of amino acids (asparagine, alanine, proline, serine) in the preculture medium also enhanced the survival rate of Dioscorea deltoidea cells after storage at low temperature.

It is evident from above cited examples that the use of pretreatment or culture conditions prior to freezing is beneficial for post-freezing survival of the cells.

C. Freezing Methods

What are the different freezing methods used for cryopreservation?

Methods used by different workers for cryopreservation of various plant materials can be categorized as - (i) slow freezing, (ii) rapid freezing and (iii) droplet-freezing.

I. Slow freezing

Methods of slowly freezing biological specimens are based on the physicochemical events occurring during the freezing. When plant cells are cooled progressively, ice crystal formation is usually initiated extra-cellularly. It is presumed that plasma membrane acts as a barrier that prevents the ice crystal formation in the cytoplasm. In absence of ice crystals, cytoplasm remains super cooled. On further lowering of temperature, the concentration of extra-cellular liquid increases, as more water is converted to ice. Since the vapour pressure of the frozen

solution is lower than the same concentration of the super cooled liquid, the vapour pressure of slowly cooled cells reach equilibrium with external ice by efflux of water. Thus, slow freezing prevents the intra-cellular ice formation and consequently freezing injury is prevented. It is believed that slow freezing increase the concentration of cytoplasm and increased dehydration increases the survival of cells. There are different methods of obtaining protective dehydration, including slow cooling at constant or varying cooling rates, or keeping the samples at one or more intermediate subzero temperatures.

The development of efficient slow freezing method depends upon several factors like:

1. Cooling rates,
2. Pretreatment and cryoprotection,
3. Type and physiological state of the material, and
4. The temperature prior to immersion in liquid nitrogen. Slow cooling method used for different species is given in Table 2. The most commonly used methods for the cryopreservation of plant cells generally involves regulated slow cooling at a constant rate of 0.5 to 2°C/min. to terminal temperature between -3°C to -40°C followed by storage in liquid nitrogen.

II. Rapid Freezing

Rapid freezing is unsuitable for the cryopteservation of cell cultures, it is employed to cryopreserve shoot tips. It has been used for preservation of shoot tips of carnation, potato, strawberry and several others (Table 3.) and somatic emboyos of oil palm.

Rapid freezing is accomplished by direct immersion of the cryoprotectant-treated specimens in liquid nitrogen. The cooling rate in this method is very high, usually several hundred degrees per minute. At such high cooling rates, the intracellular fluids do not have sufficient time to equilibrate with the external ice with the possibility of intracellular ice formation, which is considered to be lethal for cells.

Table 2. Summarised conditions for optimal cryopreservation for a few species

Plant species	Cryoprotectant	Cooling	Result
Acer pseudoplatanus	DMSO 24% Glucose 10% Glycerol 10% Storage –196 oC	Prefreezing - 30°C-50°C, Storage - 196°C Prefreezing 1oC	20-30% reduction in survival 28% cel1 survival
Atropa belladonna	DMSO %	Prefreezing 2oC/min. Storage –196 oC	40% cell survival
Datura stramonium	DMSO 5%	Prefreezing 1-2 oC/min. Till –100 oC Storage-196oC	40% cell survival
Daucus carota	DMSO 10% or Glycerol 5%	Prefreezing 2-4 oC/min Storage –196 oC	65% cell survival after 60 Days. Storage, plant Regeneration.
Ipomoea sp.	DMSO 2.5% Glycerol 2.5% Sucrose 6.5%	Pre-freezing 2-4°C/min. Storage - 196°C	5.2% survival, high growth in few days.

Table 3. Plant species in which callus, cells, protoplasts and meristem/ shoot tips have been used for cryopreservation.

Cell Culture	Callus Cultures		
Acer pseudoplatanus	Medicago sativa	Malus domestica	Meristem/ shoot tips
Atropa belladonna	Populus sp.	Medicago sativa	Arachis hypogaea
Brassica napus	Saccharum sp.	Nicotiana tabaccum	Brassica napus
Capsicum annuum	Triticum aestivum	Oryza sativa	Cicer arietinum
Catharanthus roseus	Ulmus americana	Panax ginseng	Dianthus caryophyllus
Datura innoxia	Protoplast Cultures	Populus sp.	Lycopersicon esculentum
Dioscorea deltoidea	Datura innoxia	Rose Pauls scarlet	Malus domestica
Digitalis lanata	Daucus carota	Saccharum sp.	Manihot esculenta
Daucus carota	Glycine max	Zea mays	Pisum sativum
Glycine max	Hordeum vulgare		Solanum tuberosum.
Hordeum vulgare	Triticum aestivum	Lavandu/a vera	Strawberry
Hyoesymus muticus	Zea mays	Others in various spp.	
		Somatic embryos	Pollen
		Anther	Whole seeds

iii. Droplet freezing - In this method, the cryoprotectant treated meristems are dispensed in droplets of 2-3 ml on an alluminium foil in a petriplate. The specimens are frozen by slow cooling (0.5oC/min.) to a subzero temperature between -20 to -40°C prior to immersion in liquid nitrogen.

iv. Storage, thawing and regrowth - Material can be kept stored in liquid nitrogen (-196°C) or in its vapour (-150oC). Rapid thawing is recommended for most cryopreservation methods. The basis of applying rapid thawing is to avoid the damaging ice recrystallization which may occur during slow warming. In general, thawing is carried out by removing the sample by liquid nitrogen storage and transferring in a water bath (34- 40°C) for about 1-2 min or until material is warmed up.

Regrowth of cryopreserved specimens is the most reliable and accurate estimate of viability. The other technique used for vitality test of the cells such as fluorescein diacetate stain, triphenyl tetrazolium chloride test etc. may provide a quicker method of testing cell viability. The further growth and regeneration of the cryopreserved cells after thawing will be like normal cells. However, care should be taken in handling, plating and subculture of such cells.

Recent Developments

A. Vitrification

What is the procedure followed in Vitrification?

The development of vitrification (vitreous means glass like, derived from Latin vitreus = 'glass') technique for the cryostorage of cultured plant cells and organ is of recent origin. A normal procedure involves a freeze induced cell dehydration step prior to liquid nitrogen storage. An alternate approach to cryopreservation is based on the ability of highly concentrated solutions of cryoprotectants to supercool to very low temperatures with rapid cooling, to become viscous at sufficiently low temperatures, and solidify without the formation of ice. This process is known as vitrification. If the cells are capable of tolerating this severe osmotic stress to allow glass transition to occur, theoretically they should be able to survive the freezing process. It is a very simple process and does not require costly programmable freezing equipment.

B. Encapsulation

What is Encapsulation?

Basically, this technique is used in synthetic seed technology by coating somatic embryos in alginate beads. For cryopreservation, shoot tips or embryos are coated in sodium alginate and dehydrated by placing in medium containing high concentration of sucrose (0.3-0.7 M). In this way, encapsulated material loses water by exo-osmosis. Further, drying is carried out in Laminar air flow for 2-6 hours and than transferred to liquid nitrogen. This method has been used for carrot, Pyrus communis and So/anum phureja.

C. Freezing Apparatus

What happens in Freezing Units?

Various types of cryostate and freezing units are available by which different rates of cooling can be easily regulated (Fig.2). The culture subjected to ultra cooling can be stored at -196°C in the liquid nitrogen container for various lengths of time, and can be taken out and thawed when required.

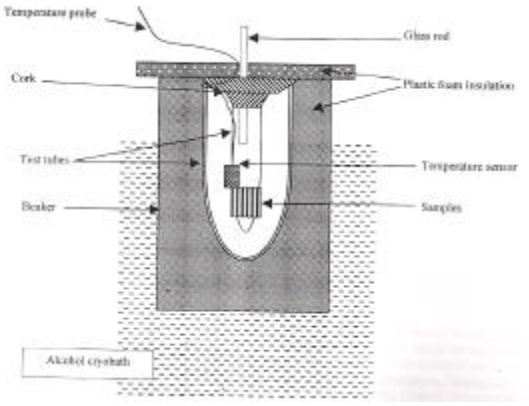

Fig. 2. Apparatus used for freezing the plant samples in cryobath for further immersion in the liquid nitrogen

Applications of Cryopreservation

1. Conservation of genetic uniformity
2. Preservation of rare genomes
3. Freeze storage of cell cultures and cell lines
4. Maintenance of disease free material.
5. Cold acclimation and frost resistance
6. Retention of morphogenetic potential in long-term cultures
7. Slow metabolism and aging.

Discussion

In spite of the information and technology available for in-vitro storage of several crop species, in vitro active gene banks exist so far only for potato at International Potato Cenre, Lima, Peru and cassava at Centro International de Agricultural Tropical (CIAT), Cali, Colombia. Recently, efforts have been made under the auspices of International Board for Plant Genetic Resources (IBPGR) to exploit the full potential of in vitro storage methods employing meristem and shoot tip cultures. The initiation of a pilot project by collaborative efforts of IBPGR and CIAT, using cassava as a model system, for assessing the potential and feasibility of establishing and running an in-vitro active gene bank is a first signiftcant step. Under the project more than 4000 clones of cassava are being maintained in-vitro under minimal growth conditions. In India, storage facility has been created at National Bureau of Plant Genetic Resources (NBPGR), New Delhi for germplasm preservation using in vitro methods.

Questions

1. Describe the methods of preservation for germplasm.
2. What is cryopreservation ? Describe the procedure and applications of cryopreservation for storage of germplasm.
3. Write short notes on the following:
   a. Germplasm
   b. Cryopreservation
   c. In situ preservation
   d. Ex situ preservation
   e. Thawing